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Identification of a regulatory motif in Hsp70 that affects ATPase activity, substrate binding and interaction with HDJ-1

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The Hsp70 family of molecular chaperones has an essential role in the synthesis, folding and translocation of the nascent peptide chain. While the general features of these activities are well documented, less is understood about the regulation of these activities. The ATPase rate is stimulated by non-native proteins, furthermore, interaction with ATP leads to the release of protein substrate concurrent with a conformational change in Hsp70. One interpretation of these data is that the two domains of Hsp70 interact. In the process of mapping the carboxyl-terminal boundary of the substrate binding domain for human Hsp70, we identified a regulatory motif, EEVD, which is conserved at the extreme carboxyl terminus among nearly all cloned cytosolic eukaryotic Hsp70s. Deletion or mutation of EEVD affects the ATPase activity, the ability to interact with substrates, and interferes with the ability of the mutant Hsp70 to interact with HDJ-1 in the refolding of denatured firefly luciferase. Examination of the biophysical properties of the mutant Hsp70s reveals a change in the overall shape and conformation of the protein consistent with reduced interactions between the two domains. These data suggest that the EEVD motif is involved in the intramolecular regulation of Hsp70 function and intermolecular interactions with HDJ-1.

Key words: chaperones/Hsp70/protein folding

Introduction

The Hsp70 family of molecular chaperones is ubiquitous and highly conserved with diverse biochemical roles in events including protein synthesis, protein translocation, protein-mediated protein folding, uncoating of clathrin-coated vesicles, protein degradation and modulating gene expression (Tilly *et al.*, 1983; Ungewickell *et al.*, 1985; Munro and Pelham, 1986; Chirico *et al.*, 1988; Murakami *et al.*, 1988; Chiang *et al.*, 1989; Beckmann *et al.*, 1990; Skowyra *et al.*, 1990; Stone and Craig, 1990; Abravaya *et al.*, 1992; Nelson *et al.*, 1992; Shi and Thomas, 1992). In humans, the Hsp70 multi-gene family includes the cytosolic and nuclear localized Hsc70 and Hsp70, endoplasmic reticulum localized Grp78 (BiP) and mitochondrial localized MtHsp75 (Grp75) (Hunt and Morimoto, 1985; Wu *et al.*, 1985; Dworniczak and Mirault, 1987; Ting and Lee, 1988; Bhattacharyya *et al.*, 1995). Hsc70, MtHsp75 and Grp78 are abundantly expressed during normal growth conditions and the level of each protein is induced to various extents in response to specific types of stress. In contrast, the levels of Hsp70 are growth regulated (Wu *et al.*, 1985; Milarski and Morimoto, 1986) and strongly induced by a variety of conditions of physiological and environmental stress (Morimoto *et al.*, 1992).

The 70 kDa chaperones contain two functional domains, an amino-terminal 45 kDa ATP binding domain and a carboxyl-terminal 25 kDa substrate binding domain. The crystal structure of the 45 kDa fragment of bovine Hsc70 reveals a bi-lobed domain with a deep cleft in which the ATP molecule is bound (Flaherty *et al.*, 1990). The carboxyl domain is sufficient and necessary for substrate binding, however, regulation of the substrate binding activity does not occur in the absence of the ATP binding domain (Chappell *et al.*, 1987; Milarski and Morimoto, 1989; Haus *et al.*, 1993; Tsai and Chang, 1994). An indication that sequences other than the ATP binding and substrate binding domains are important was provided by the analysis of a 60 kDa fragment of bovine Hsc70 (amino acids 1–543) which could bind to clathrin cages, yet was incapable of stimulating disassembly of the clathrin cage (Chappell *et al.*, 1987). The ATPase activity of the 60 kDa fragment of human Hsc70 was stimulated by protein substrate, indicating that both activities were present, but perhaps not regulated properly (Tsai and Chang, 1994).

It is generally considered that the diverse activities of Hsp70s reflect their primary role in association with nascent peptide chains and non-native proteins (Craig *et al.*, 1994; Frydman and Hartl, 1994; Georgopoulos *et al.*, 1994; Hightower *et al.*, 1994). Although there are reports that Hsp70s can function alone to prevent protein aggregation and assist in the refolding of denatured substrates, members of the Hsp70 family often require interactions with specific members of the co-chaperone DnaJ family for efficient protein folding and translocation (Caplan *et al.*, 1993). Following these initial events, import of nascent chains into the mitochondria requires the mitochondrial homologs of Hsp70 (mtHsp75)–DnaJ and subsequent interactions with Hsp60 (GroEL) to acquire the final folded state (Ostermann *et al.*, 1989; Kang *et al.*, 1990). Likewise, in the lumen of the endoplasmic reticulum, the Hsp70 homolog (Grp78) associates with the translocated nascent chain. The events involved in the folding of cytosolic proteins are less well characterized. TCP-1 has been identified as a cytosolic homolog to GroEL (Lewis *et al.*, 1992), however, only a few proteins

(β-actin, α- and β-tubulin) are known to require this chaperone for folding (Gao *et al.*, 1992; Yaffe *et al.*, 1992).

In this study we have addressed the question of how the ATPase and substrate binding domains of the cytosolic Hsp70s are regulated. We have identified an acidic motif, EEVD, located at the extreme carboxyl terminus of nearly all Hsp70s which has an essential role in regulating the biochemical properties of Hsp70 and interactions with HDJ-1.

Results

Identification of the sequences required for substrate recognition: deletion from the carboxyl terminus of Hsp70 interferes with substrate recognition

The ability of recombinant human Hsp70 to distinguish between native α-lactalbumin and reduced carboxymethylated lactalbumin (RCMLA) as substrates was examined using gel filtration (Figure 1; Palleros *et al.*, 1991). RCMLA and α-lactalbumin were labeled with ^{125}I, incubated with wild type or mutant Hsp70 protein, and the bound and free populations of α-lactalbumin or RCMLA were separated by chromatography. Gel filtration of either protein alone yields a single symmetric peak, whereas ~50% of the RCMLA elutes as a RCMLA–Hsp70 complex following incubation with wild type Hsp70 (Figure 1). Wild type Hsp70 does not form a stable complex with α-lactalbumin which demonstrates that Hsp70 binds preferentially to the non-native protein.

The location of the Hsp70 substrate binding domain was mapped using deletion mutants of human Hsp70. The boundaries of the Hsp70 mutants are shown schematically in Figure 2A and the homogeneity of the purified recombinant proteins is indicated in Figure 2B. Comparison of the binding data for the mutant Hsp70s reveals that deletion within the carboxyl domain severely reduces RCMLA binding activity, whereas loss of the residues in the amino terminus does not diminish substrate binding (Figure 2A). Comparison of mutant ΔNSBt (residues 415–640) which binds substrate, and ΔNSSmat (residues 437–640) which cannot, establishes the amino-terminal boundaries of the substrate, binding domain between amino acids 415 and 436. Three mutants (ΔSmat, ΔBCt and ΔCRt) containing internal carboxyl-terminal deletions were unable to bind to RCMLA, whereas two mutants (386–640t and ΔNSBt) retaining the corresponding sequences in the carboxyl terminus bind to substrate (Figure 2C). To establish the carboxyl-terminal boundary, we examined deletion mutants with carboxyl termini at residues 543, 567 or 611. As shown in Figure 2C and summarized in Figure 2A, loss of any carboxyl-terminal sequence beyond residue 611 resulted in the complete loss of binding activity. However, a small internal deletion within the extreme carboxyl terminus (Δ616–625) had no apparent effect on the substrate binding activity. These data reveal that the carboxyl terminus is necessary and sufficient for substrate binding.

Deletion of the EEVD residues affects the ability of Hsp70 to bind to RCMLA

The results of carboxyl-terminal deletion analysis suggest that sequences between residues 626 and 640 are important

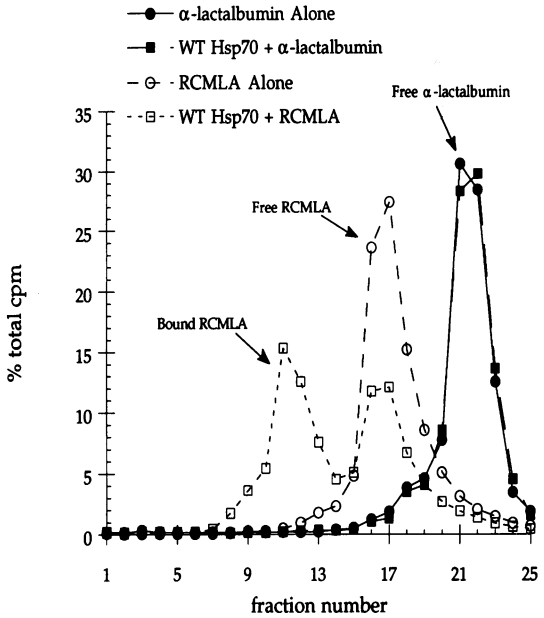

Fig. 1. Wild type Hsp70 specifically binds to the ^{125}I-radiolabeled non-native form of α-lactalbumin. Samples containing α-lactalbumin alone, Hsp70 + α-lactalbumin, RCMLA alone, or Hsp70 + RCMLA were incubated prior to resolution over a gel filtration column. The data are presented as per cent of total substrate in the reaction.

for Hsp70 function. Therefore we examined the amino acid sequences in this region of ~46 members of the Hsp70 family for which nucleotide sequence information is available. Of these, 36 sequences, corresponding to cytosolic Hsp70s from diverse eukaryotic species, had the terminal acidic motif, EEVD (Figure 3). To examine the potential role of EEVD in Hsp70 function, we generated a collection of deletion and point mutants in the EEVD sequence to study the biochemical properties of the purified recombinant proteins (Figure 4).

Comparison of the substrate binding properties of the wild type and mutant Hsp70s revealed that deletion of EEVD or alanine substitution (AAAA) of EEVD had a striking negative effect (Figure 4A). Substitution of both glutamic acids residues (AAVD) reduces the fraction of bound RCMLA to near background levels (Table I). The inability of these mutant Hsp70 proteins to bind RCMLA does not appear to be simply due to the net loss of two negatively charged residues as the mutant AEVA retained the ability to bind to substrate (Table I and Figure 4B). The substrate binding results were corroborated using a native gel shift assay in which the complex between the radiolabeled RCLMA and Hsp70 is detected on a native acrylamide gel. Consistent with the results obtained by gel filtration, radiolabeled RCMLA formed a stable complex with wild type Hsp70 and not with ΔEEVD, AAAA or AAVD (Figure 4B). Single non-conservative substitutions of EEVD reduced the fraction of RCMLA bound by 25% except for substitution of the second glutamic acid residue in EEVD which reduced binding by 50% (Table I). The selectivity of the mutant phenotype was revealed by the mutants EEAA, AEVA, EAVD, AEVD and EEKD, all of which retained the ability to bind to RCMLA as measured by either binding assay (Figure 4 and Table I). Additionally, the results of the gel shift assay corroborated the results of the gel filtration assay on the inability of

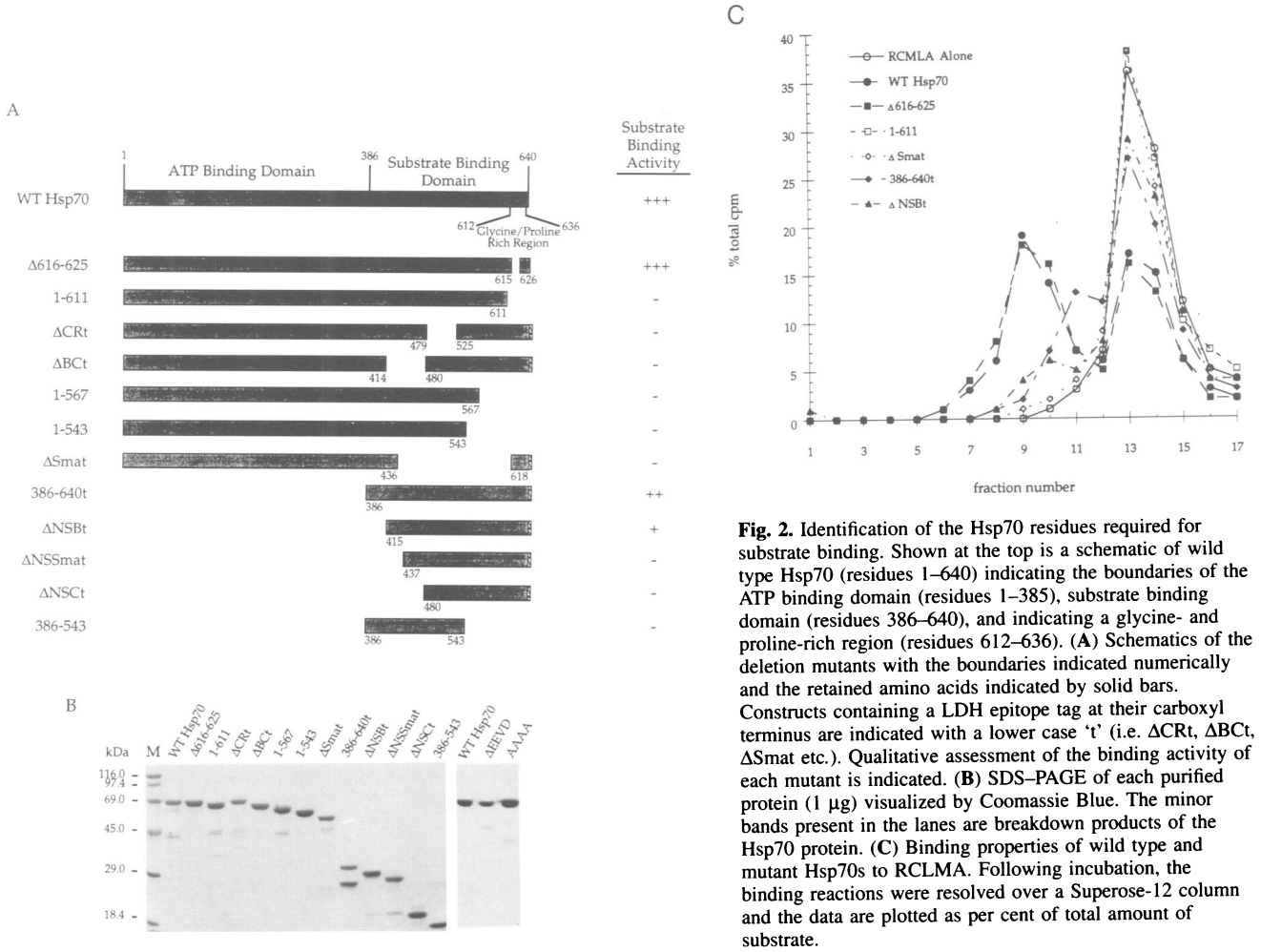

Fig. 2. Identification of the Hsp70 residues required for substrate binding. Shown at the top is a schematic of wild type Hsp70 (residues 1–640) indicating the boundaries of the ATP binding domain (residues 1–385), substrate binding domain (residues 386–640), and indicating a glycine- and proline-rich region (residues 612–636). (**A**) Schematics of the deletion mutants with the boundaries indicated numerically and the retained amino acids indicated by solid bars. Constructs containing a LDH epitope tag at their carboxyl terminus are indicated with a lower case 't' (i.e. ΔCRt, ΔBCt, ΔSmat etc.). Qualitative assessment of the binding activity of each mutant is indicated. (**B**) SDS–PAGE of each purified protein (1 μg) visualized by Coomassie Blue. The minor bands present in the lanes are breakdown products of the Hsp70 protein. (**C**) Binding properties of wild type and mutant Hsp70s to RCLMA. Following incubation, the binding reactions were resolved over a Superose-12 column and the data are plotted as per cent of total amount of substrate.

the carboxyl deletion mutants, ΔSmat, 1–543, 1–567 and 1–611 to bind to RCMLA (Figure 4B).

To examine whether the role of EEVD in substrate binding was unique to inducible Hsp70, we compared the binding properties of the constitutively expressed human Hsc70 with that of two carboxyl-terminal deletion mutants of the corresponding EEVD residues in Hsc70. As shown in Figure 5, deletion of EEVD (c1–642) in Hsc70 led to a 3-fold reduction in RCMLA bound. Further truncation to residue 544 (c1–544) had no apparent additional effect on the substrate binding activity in comparison to the EEVD deletion mutant. Although the results using the RCMLA binding assay are somewhat less dramatic for Hsc70 than with Hsp70, taken together, they offer support for the involvement of the EEVD motif in substrate binding.

Alteration or deletion of EEVD alters the ATPase activity

Another common feature of Hsp70s is ATP binding and ATP hydrolysis (~3 pmol/min/μg of protein; Sadis and Hightower, 1993). The intrinsic ATPase is enhanced upon interaction with malfolded protein substrates. Relative to the ATPase activity of wild type Hsp70, deletion or non-conservative substitution of both glutamic acids (AAAA and AAVD) has a 1.5- to 2.5-fold stimulatory effect (Table I). Substitution of the aspartic acid residue (EEAA and AEVA) slightly decreased the ATPase rate relative to wild type. We also examined the peptide-stimulated ATPase and found that RCMLA had a stimulatory effect even on those mutants (ΔEEVD, AAVD and AAAA) that did not form a stable complex with RCMLA, as tested by gel filtration or native gel electrophoresis. Taken together, these data reveal that complete deletion of EEVD and complete (AAAA) or partial alanine substitution of these residues (AAVD) leads to a severe loss in substrate binding and an enhanced ATPase activity. In contrast to these severe phenotypes, substitution of single amino acids has a much weaker phenotype.

Mutations in EEVD affect the overall conformation of Hsp70

One interpretation of the previous data on ATPase rates and substrate-recognition properties is that the EEVD motif could have a role in regulating both activities. This could be accomplished if the EEVD motif influenced the overall conformation of Hsp70 and consequently affected activities of both domains. To address this, we used a combination of biochemical and biophysical approaches to probe the conformation of the native wild type and mutant Hsp70s.

The fluorescence emission spectrum of tryptophan residues can provide a measure of the conformational state of a protein. This method can detect subtle changes in the conformation of a protein as long as the change(s) include the area surrounding the tryptophan. Hsp70 has

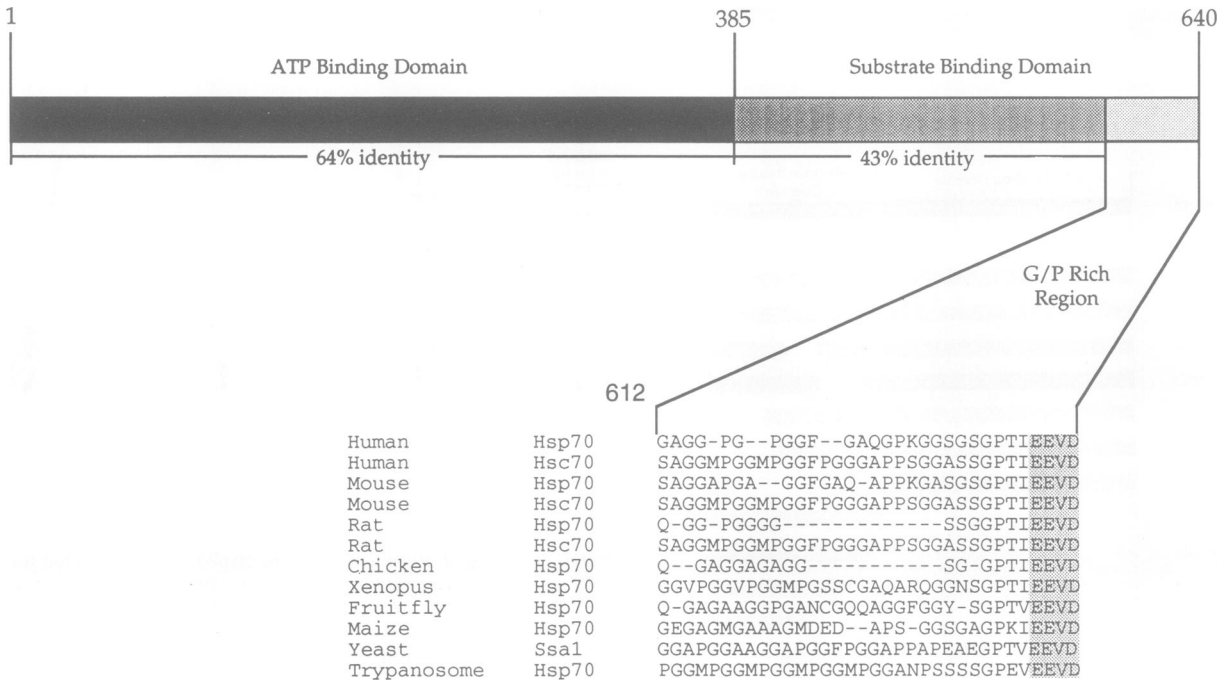

Fig. 3. Conserved features in the carboxyl terminus common to eukaryotic Hsp70s. Shown below the schematic of wild type Hsp70 and the boundaries for ATP- and substrate binding domain is the expanded sequence for the glycine- and proline-rich region extending from residue 612. The sequences for trypanasome Hsp70 (Requena *et al.*, 1988), yeast Ssa1 (Slater and Craig, 1989), maize Hsp70 (Rochester *et al.*, 1986), fruitfly Hsp70 (Ingolia and Craig, 1982), *Xenopus* Hsp70 (Bienz, 1984), chicken Hsp70 (Morimoto *et al.*, 1986), rat Hsp70 (O'Malley *et al.*, 1985), rat Hsc70 (O'Malley *et al.*, 1985), mouse Hsp70 (Hunt and Calderwood, 1990), mouse Hsc70 (Giebel *et al.*, 1988), human Hsp70 (Wu *et al.*, 1985) and human Hsc70 (Dworniczak and Mirault, 1987) are indicated. The EEVD motif is indicated as shaded.

two tryptophan residues at position 78 in the ATP binding domain and at position 579 in the carboxyl-terminal domain. The tryptophan fluorescence emission spectrum was determined for four selected proteins (wild type, ΔEEVD, AEVD and AAAA). Wild type Hsp70 exhibits a maximum absorbance at 334 nm whereas the ΔEEVD and AAAA mutant proteins exhibited decreased fluorescence and a shift to 332 nm in their maxima (Figure 6). While our data reflect the sum absorbance properties of both tryptophans, these data suggest that the conformation of the mutant proteins is altered.

The Stokes' radii and frictional ratios for the wild type and mutant proteins were determined to obtain independent measures of the conformation of Hsp70 (Table I). The AEVD protein had a slightly larger Stokes' radius and frictional ratio (f/f_o) than wild type. The Stokes' radius and f/f_o increased from 44 Å and 1.58, as measured for the wild type, to 51 Å and 1.83 for the ΔEEVD protein. The changes in the physical properties of the mutants correlate with the functional data for the various proteins; the ΔEEVD and AAAA proteins exhibited similar properties and the properties for the AEVD protein were intermediate between the wild type and ΔEEVD or AAAA proteins.

The conformation of the wild type and mutant Hsp70s was also examined through the use of limit proteolytic digestion (Deluca-Flaherty *et al.*, 1990; Liberek *et al.*, 1991a). We first examined the proteolytic sensitivity of wild type Hsp70 in the presence of trypsin (Figure 7A, upper left panel). The stability of the full length wild type Hsp70 could be enhanced by the addition of either ATP (upper right panel) or RCMLA (lower right panel), however, addition of α-lactalbumin (lower left panel) had no apparent effect, as quantified and presented in Figure 7A. Digestion of the ΔEEVD protein under the same conditions (Figure 7B) revealed that the mutant protein was more rapidly hydrolyzed, yet the addition of ATP or substrate resulted in an increased stability. We next compared the protease sensitivity of the mutants AEVD and AAAA and observed that the kinetics of degradation of AAAA protein were comparable to ΔEEVD and that the kinetics of the AEVD were intermediate between the wild type, ΔEEVD and AAAA proteins. These results, together with the ATPase data, support the conclusion that the ΔEEVD protein interacts with RCMLA despite its inability to form stable complexes with RCMLA.

To examine whether the increased protease sensitivity exhibited by the EEVD mutants is due to misfolding of either the ATP- or substrate binding domain, we used monoclonal antibodies to Hsp70 specific to either the amino (5A5) or carboxyl (3A3) terminus to probe Western blots of protease-treated wild type and ΔEEVD protein. Under these conditions of trypsin digestion, wild type Hsp70 was essentially unaffected (Figure 8, top left, lower left panels). In contrast, the ΔEEVD protein was digested to a stable 45 kDa amino-terminal fragment (upper right panel) and 25 kDa carboxyl fragment (lower right panel). These data reveal that the ΔEEVD protein exhibits an increased digestion with trypsin at a cleavage site located between the 45 kDa amino and 25 kDa carboxyl-terminal domains. These results suggest that the conformational difference(s) between the wild type Hsp70 and ΔEEVD protein are not specifically localized to either the amino or carboxyl domain but rather that the intra-domain interaction(s) have been altered.

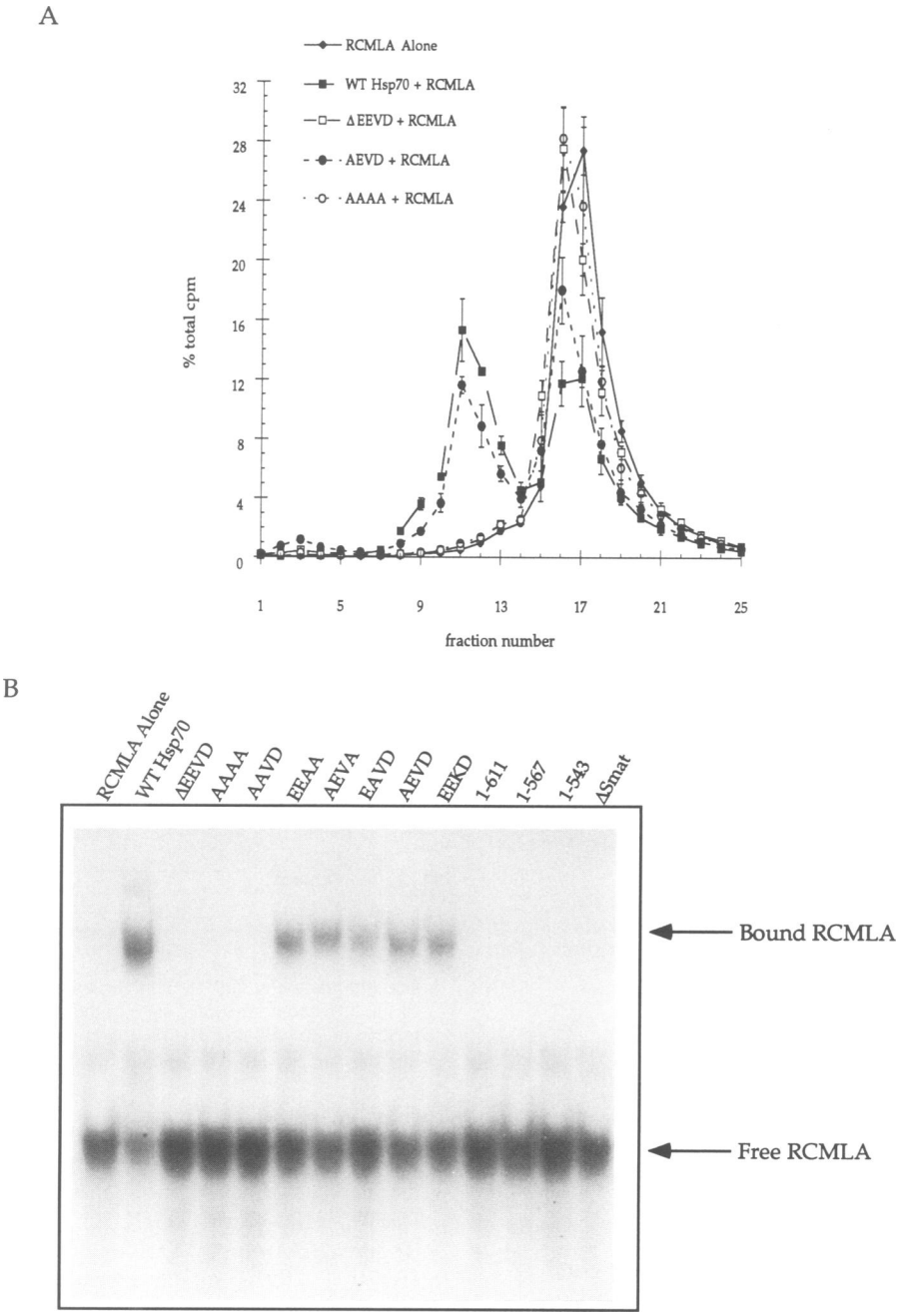

Fig. 4. Mutations in the EEVD motif of Hsp70 affect substrate binding. (**A**) Binding properties of wild type and mutant Hsp70s. Comparison of RCLMA alone, wild type Hsp70 + RCMLA, ΔEEVD + RCMLA, AEVD + RCMLA, and AAAA + RCMLA. (**B**) Analysis of RCLMA binding to wild type and mutant Hsp70s using a native gel electrophoresis assay. The free and bound iodinated RCMLA is indicated.

The EEVD motif couples substrate recognition and the ATPase domain

To examine whether the inability of mutant Hsp70 to form a stable complex with RCMLA is due to a direct requirement for EEVD in substrate binding or alternatively, that EEVD interacts with the ATP binding domain, we determined the substrate binding activities of wild type and mutant Hsp70s lacking the ATP binding domain. The carboxyl terminus (residues 386–640) of wild type or mutant (AAVD or AAAA) Hsp70 was overexpressed, purified and used for the gel filtration substrate binding assay. As shown in Figure 9, the wild type and mutant Hsp70 carboxyl-terminal domains bind equally well to RCMLA, in contrast to the results obtained with the mutant Hsp70s containing the ATP binding domain (Table I; Figure 4A). One interpretation of these data is that the ability of EEVD to modulate binding of substrate or access to the substrate-recognition domain is influenced by the ATP binding domain. These data, in conjunction with the tryptic digestion data, suggest that alteration of the EEVD residues does not directly affect the substrate binding domain but rather alters the interaction between the amino and carboxyl domains.

Finally, our ability to perform substrate binding studies with carboxyl-terminal fragments of Hsp70 lacking the ATP binding domain would suggest that it should be

Table I. Summary of substrate binding properties, intrinsic ATPase, substrate-stimulated ATPase activity and biophysical properties of the WT and mutant Hsp70s

Protein	% RCMLA bound	ATPase rate (pmol/min/μg)		Stokes' radii (Å)	f/f_0
		Alone	+RCMLA		
WT Hsp70	44 ± 4	2.94 ± 0.25	4.20 ± 0.16	44 ± 0.5	1.58 ± 0.08
ΔEEVD	3 ± 1	4.45 ± –0.36	7.29 ± 0.33	51 ± 0.5	1.83 ± 0.11
AEVD	32 ± 4	3.08 ± 0.12	4.77 ± 0.39	46 ± 0.5	1.66 ± 0.07
EEKD	33 ± 3	2.78 ± 0.23	3.92 ± 0.22	ND	ND
EAVD	24+ ±	2.74 ± 0.15	3.74 ± 0.22	ND	ND
EEAA	34 ± 3	1.93 ± 0.18	2.87 ± 0.26	ND	ND
AEVA	28 ± 3	2.31 ± 0.20	4.20 ± 0.57	ND	ND
AAVD	2 ± 1	7.53 ± 0.36	11.45 ± 0.92	ND	ND
AAAA	3 ± 1	7.21 ± 0.64	10.63 ± 0.84	52 ± 0.5	1.87 ± 0.09

ND–not determined.

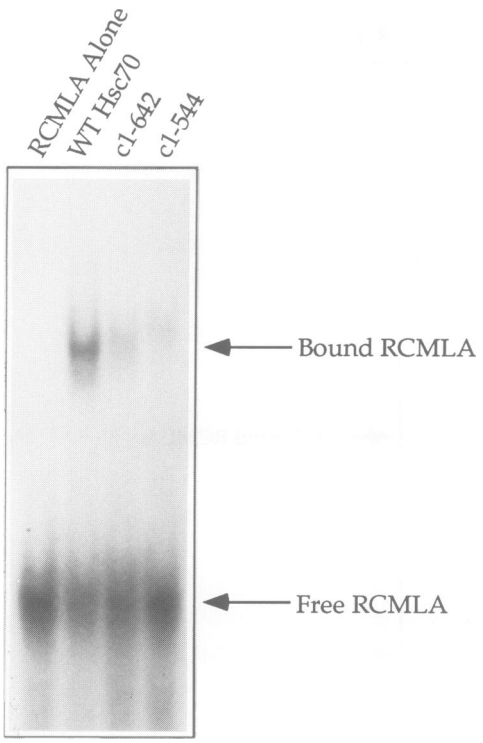

Fig. 5. Mutations in the EEVD motif of Hsc70 affect substrate binding. The substrate binding activity of wild type human Hsc70 compared with c1–642 (ΔEEVD Hsc70) and c1–544 (carboxyl-terminal deletion of 545–646) was measured using the native gel electrophoresis assay. The free and bound iodinated RCMLA is indicated.

possible to analyze the substrate binding properties of other deletion mutants. Carboxyl domain fragments retaining residues 386–578 or 386–567 did not bind RCMLA using either gel filtration or native gel electrophoresis, and the recombinant protein retaining residues 386–611 is synthesized as an insoluble aggregate.

EEVD is required for Hsp70 function in luciferase refolding

We assessed the biochemical consequence of the reduced ability of the Hsp70 mutants, ΔEEVD and AAAA, to form a stable substrate complex using another assay which measures the reactivation of denatured firefly luciferase in the presence of the heat shock proteins, Hsp70 (Hsc70)

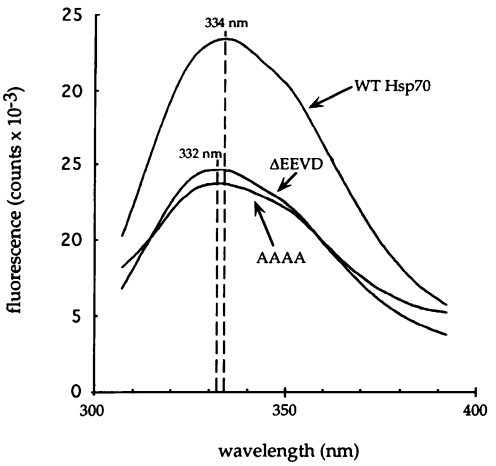

Fig. 6. Mutation of EEVD alters the tryptophan fluorescence emission spectra. The conformational state of the ΔEEVD and AAAA proteins relative to the wild type Hsp70 was examined utilizing tryptophan fluorescence emission. The maxima of the spectra are indicated.

and HDJ-1 (human DnaJ homolog; Raabe and Manley, 1991). As shown in Figure 10, luciferase activity was not recovered by the addition of wild type human Hsp70, Hsc70, HDJ-1 or DnaK in the presence of ATP (Figure 10A) nor by various combinations of Hsp70 or Hsc70 + HDJ-1 in the absence of ATP (Figure 10B). However, the addition of either Hsp70 or Hsc70 together with HDJ-1 and ATP reactivated denatured luciferase as measured by the time-dependent reappearance of luciferase activity (Figure 10C and D). In contrast to the ability of wild type Hsp70 or Hsc70 to reactivate luciferase, the ΔEEVD or AAAA mutants were non-functional in this assay. These results corroborate the RCMLA binding data; furthermore they provide direct functional evidence that the EEVD residues have an important role in Hsp70 activity.

These results also indicate the specificity of HDJ-1 interaction between Hsp70 and Hsc70 in the cooperative refolding of denatured firefly luciferase. Despite an apparent equivalent RCMLA binding activity (Figures 4 and 5) refolding of luciferase was both kinetically faster and more efficient with Hsc70 and HDJ-1 than with Hsp70 and HDJ-1. Specificity of interaction with HDJ-1 is also evident in the inability of DnaK and HDJ-1 to refold luciferase.

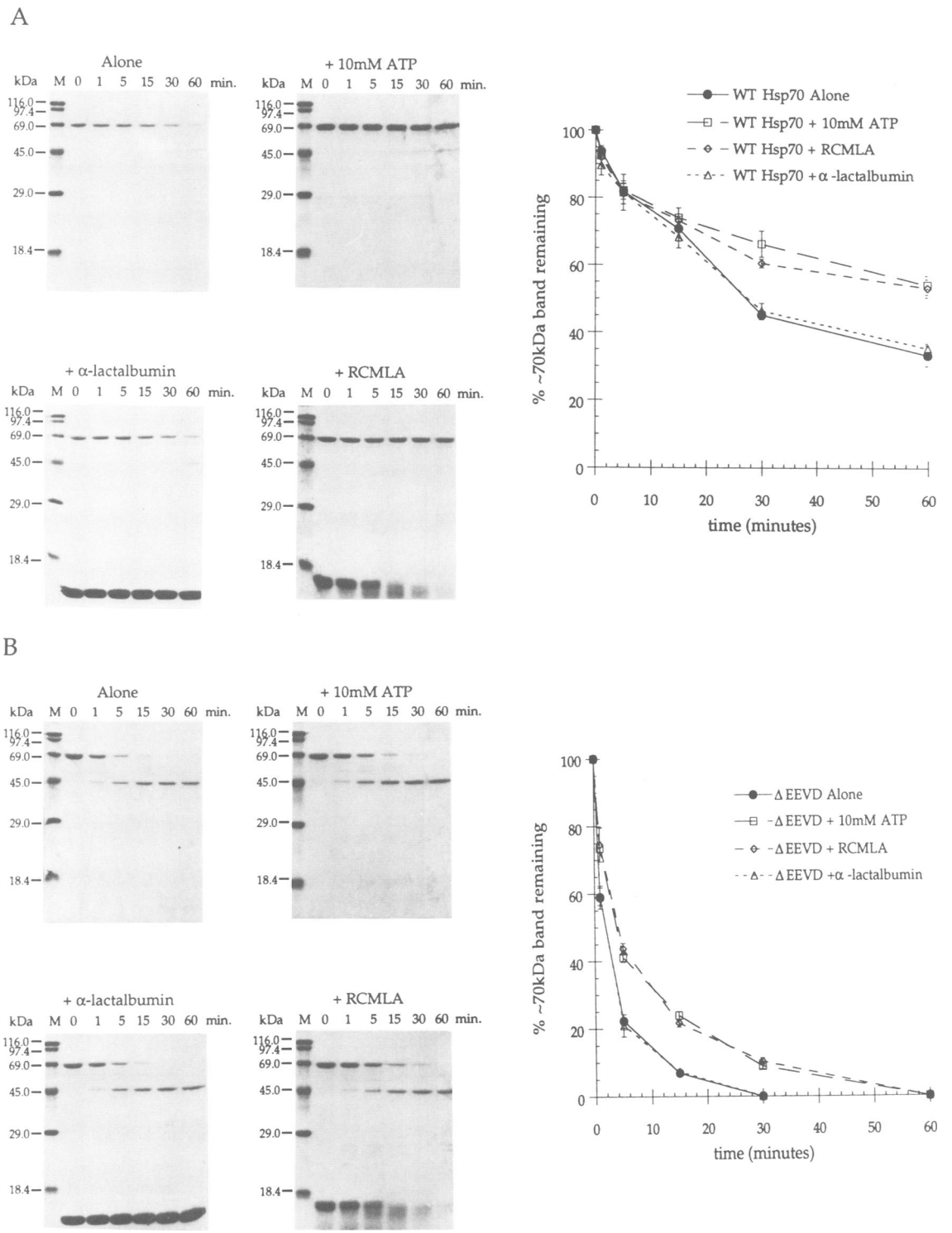

Fig. 7. The ΔEEVD mutant is protease sensitive. The stability of either wild type Hsp70 (**A**) or ΔEEVD (**B**) protein in the presence or absence of ATP, α-lactalbumin or RCMLA was determined utilizing limit trypsin digestion. Following protease treatment the products were resolved by SDS–PAGE and visualized by Coomassie Blue. The full length wild type or ΔEEVD protein coresponding to the undigested protein was quantified by laser densiotometry.

The EEVD motif is required for stimulation of the ATPase activity by HDJ-1

The evidence that the EEVD motif is essential for the Hsp70- and HDJ-1-dependent refolding of luciferase suggests that HDJ-1 does not interact with certain mutants of Hsp70. Therefore, we examined the effect of HDJ-1 on the ATPase rate of wild type and mutant Hsp70s based on the properties of the *Escherichia coli* homolog, DnaJ, which is known to stimulate the ATPase activity of DnaK above its intrinsic rate (Liberek *et al.*, 1991b). The addition of increasing concentrations of HDJ-1 stimulated the ATPase activity of wild type Hsp70 and the AEVD mutant

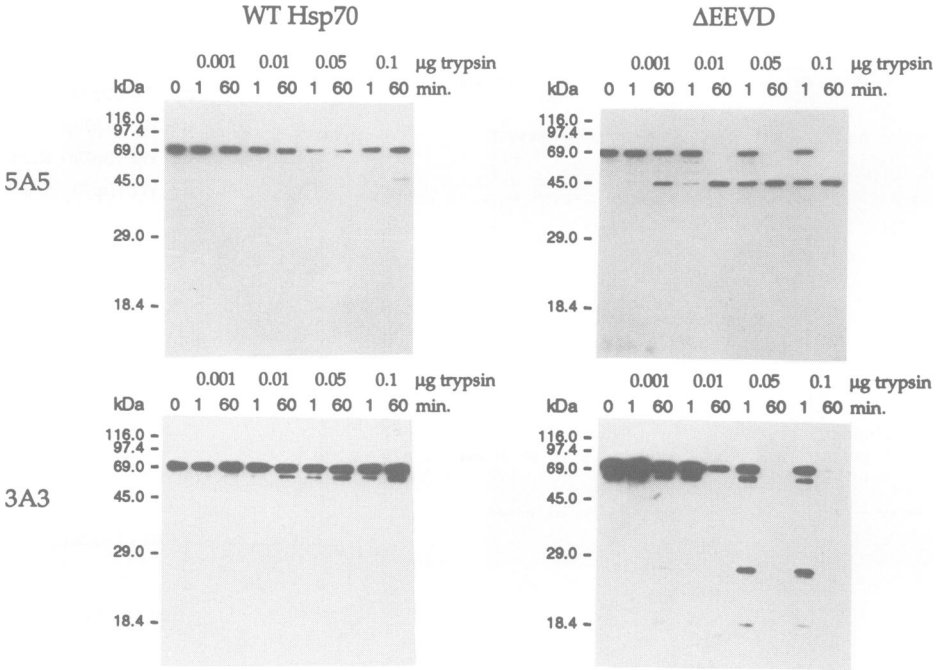

Fig. 8. The conformation of the ΔEEVD protein is affected at the hinge region separating the ATP and substrate binding domains. Trypsin digestion products of the wild type Hsp70 (left panels) or ΔEEVD (right panels) were subjected to Western blot analysis using monoclonal antibodies specific for either the amino (5A5; upper panels) or carboxyl terminus (3A3; lower panels). The lower intensity of the ~70 kDa band present in the upper left panel (0.05 µg trypsin lanes) is due to incomplete transfer. The predominant proteolytic product detected by antibody 5A5 in ΔEEVD protein is 45 kDa and by antibody 3A3 is 25 kDa.

by a factor of 2.5-fold, whereas the ATPase activity of the ΔEEVD or AAAA mutants was unaffected by HDJ-1 (Figure 11). These results parallel the phenotype of the same mutants in the luciferase refolding assays and suggests that the EEVD residues of Hsp70 may be involved in interaction with the co-chaperone HDJ-1.

Discussion

The analysis of mutations in human Hsp70 has identified a motif located at the extreme carboxyl terminus that is essential for intramolecular coupling of ATP and substrate binding activities and for intermolecular interactions between Hsp70 and HDJ-1. The functional properties of the EEVD motif appear to be common to both Hsp70 and Hsc70 which lends additional support for its role in regulating the activities of eukaryotic cytosolic Hsp70s. A number of observations suggest an interdependence of the amino- and carboxyl-terminally localized activities of Hsp70s. In the presence of nucleotide, the non-native protein substrate is released (Flynn *et al.*, 1989); likewise, the ATPase is stimulated by binding to the substrate. As we have shown here, deletion or alanine substitution of the EEVD motif affects the activities in the ATP- and substrate binding domains. Although our data does not identify the sites of intramolecular interaction for EEVD, an interaction between EEVD and the ATP binding domain seems rational. Additional evidence to support a role for EEVD as a *cis*-regulatory sequence in Hsp70 follows from the substrate binding studies of the EEVD mutants. While stable substrate interactions do not occur for the EEVD deletion or alanine substitution mutants which have the ATP binding domain, mutant Hsp70s altered in the EEVD motif and lacking the ATP binding domain can bind to

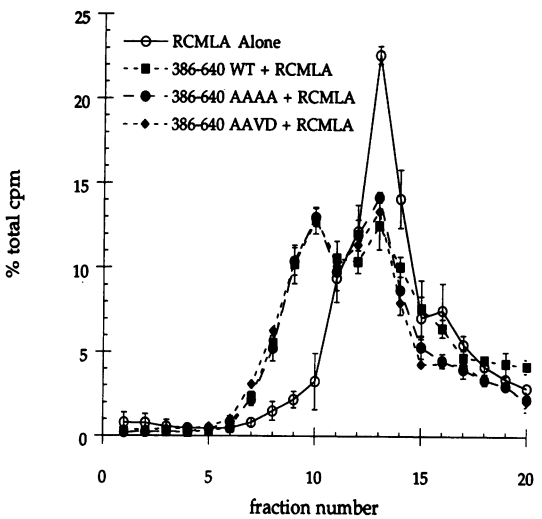

Fig. 9. The EEVD mutant (AAAA substitution) protein binds to substrate in the absence of the ATP binding domain. RCMLA binding activities of wild type and two mutant (AAVD and AAAA) recombinant proteins of the carboxyl domain (amino acids 386–640) were determined by gel filtration. The percentage of total labeled RCMLA in each fraction is indicated.

substrate. This proposed interaction between the EEVD motif and the ATP binding domain is further supported by the trypsin digestion studies of the EEVD mutants which show that the hinge region separating the ATP and substrate binding domains is more accessible.

In addition to the intrinsic activities of substrate- and ATP binding, the Hsp70s interact with other heat shock proteins and molecular chaperones. For example, in *E.coli*, proteins that stimulate the ATPase activity of DnaK include

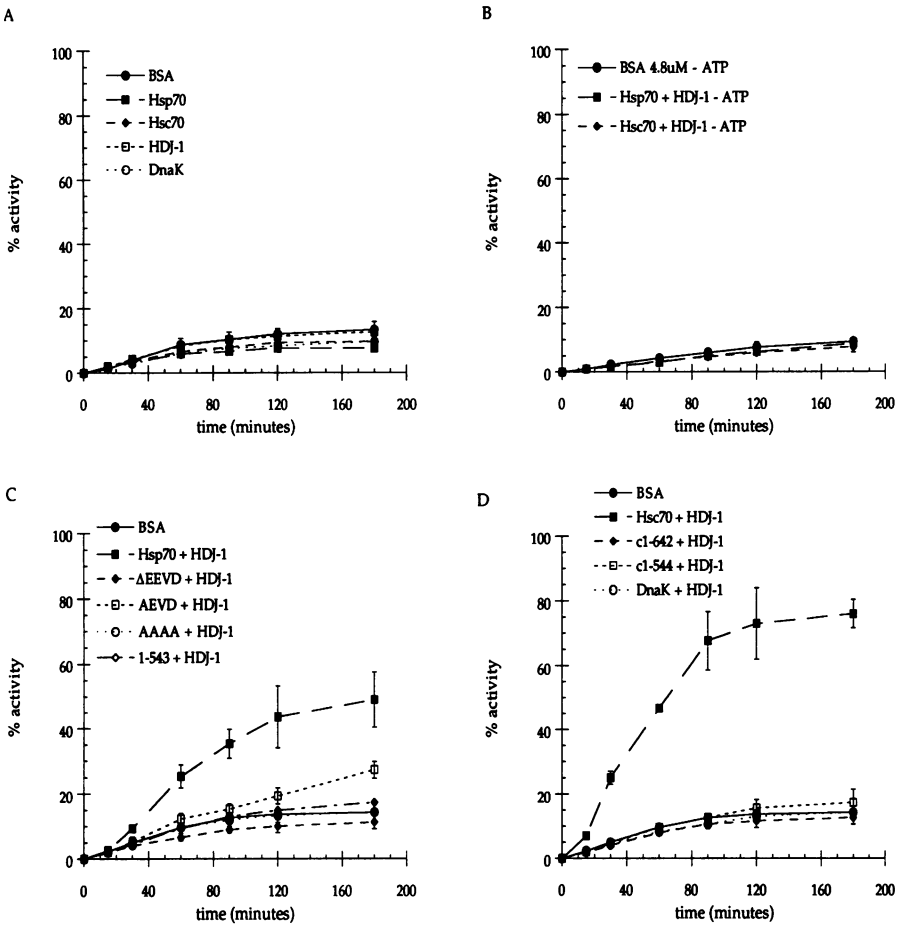

Fig. 10. The EEVD motif is required by Hsp70 for reactivation of firefly luciferase. The per cent recovered activity of firefly luciferase in the presence of individual chaperones/co-chaperone in the absence or presence of ATP was calculated utilizing an equivalent amount of non-denatured luciferase. (**A**) Effect of BSA, Hsp70, Hsc70, HDJ-1 and DnaK on luciferase activity in the presence of ATP. (**B**) Effect of BSA, Hsp70 + HDJ-1 and Hsc70 + HDJ-1 on luciferase activity in the absence of ATP. (**C**) Effect of BSA, Hsp70 + HDJ-1, ΔEEVD + HDJ-1, AEVD + HDJ-1, AAAA + HDJ-1 and 1–543 + HDJ-1 on luciferase activity in the presence of ATP. (**D**) Effect of BSA, Hsc70 + HDJ-1, c1–642 + HDJ-1, c1–544 + HDJ-1 and DnaK + HDJ-1 on luciferase activity in the presence of ATP.

DnaJ and GrpE (Liberek *et al.*, 1991b). Homologs to DnaJ are ubiquitous in eukaryotes, however, eukaryotic homologs to GrpE have only recently been identified in mitochondria (Laloraya *et al.*, 1994). The mechanism by which the DnaJs interact with Hsp70s has not been established. The evidence presented here reveals that Hsp70s deleted or substituted with alanine in EEVD cannot participate with HDJ-1 either to stimulate the ATPase rate or in the refolding of denatured luciferase. These data suggest that the EEVD motif may be involved in the mechanism by which Hsp70 and HDJ-1 functionally interact.

How are the EEVD residues involved in the regulation of Hsp70 function? One possibility is that EEVD has a key role in the transition of the unliganded state of Hsp70 to the ligand (ATP or substrate) state. Binding to either site would affect the conformation of Hsp70 and consequently access of the EEVD motif for association with HDJ-1. The EEVD motif could alternate between intramolecular interactions with the ATP binding domain and intermolecular interactions with HDJ-1. This would allow the cytosolic Hsp70s to adopt different states of substrate interaction according to subsequent events with ATP and HDJ-1 (Cyr *et al.*, 1992). Extending the period of interaction between a substrate and the 70 kDa chaperone may be useful during protein synthesis and protein translocation, and in particular during exposure to physiologically stressful conditions (Beckmann *et al.*, 1990). All of these conditions have in common the requirement that the substrate–chaperone complex is maintained until the protein is to be folded to its final conformation, translocated, or until the physiological state of the cell is less stressful. An example of a well-studied heat shock protein complex is the progesterone receptor, in which the receptor protein is maintained in an inert complex with Hsp90, Hsp70 and other proteins until the steroid is provided (Bresnick *et al.*, 1988; Smith *et al.*, 1990). Hsp90 also has the carboxyl-terminal EEVD motif and has been suggested to interact with DnaJ homologs in *S.cerevisiae* (Y.Kimura, personal communication).

Finally, in revisiting the question of the boundary of the substrate binding domain of Hsp70, our results establish a requirement for sequences that extends well beyond the regions predicted to share structural similarities with the MHC molecule (Flajnick *et al.*, 1991). In contrast, the substrate binding domain of Hsc70, while dependent on the EEVD motif, is less dependent as indicated by our data and previous observations (Chappell *et al.*, 1987;

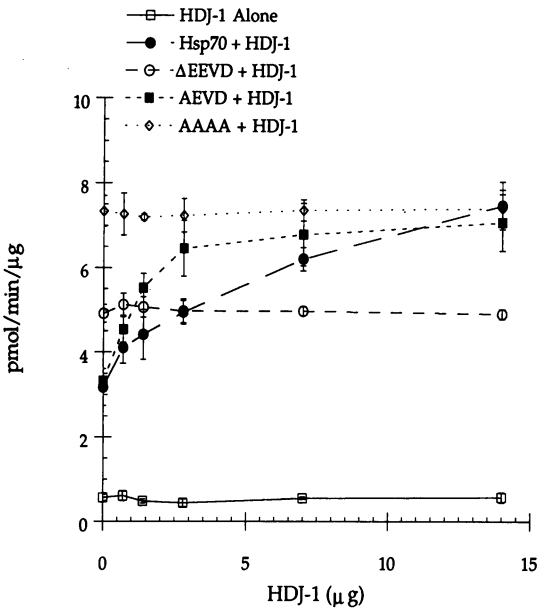

Fig. 11. HDJ-1 does not stimulate the ATPase activity of the ΔEEVD and the AAAA mutants. The ATPase rates of wild type and mutant Hsp70 proteins were determined in the absence or presence of increasing concentrations of HDJ-1. The specified amounts of HDJ-1 correspond to Hsp70:HDJ-1 molar ratios of 0, 2:1, 1:1, 1:2, 1:5 and 1:10.

Tsai and Chang, 1994). Thus, for Hsp70, one possibility is that the carboxyl domain contains a structural component for substrate recognition and a separate region to regulate these interactions. Further studies will be required for a complete understanding of the binding and regulatory properties of Hsp70 and Hsc70 with substrates, interactions with nucleotides, and the nature of interactions with HDJ-1 leading to stimulation of nucleotide hydrolysis and in the refolding of non-native proteins.

Materials and methods

Plasmid constructions

The construction of pETWThsp70, pETΔSmat and pGEXΔNSCt has been described (Abravaya *et al.*, 1992). Plasmids pETΔBCt, ΔCRt, Δ616–625, ΔNSSmat and ΔNSBt were created by digestion at existing restriction endonuclease sites, mutants 1–543, 386–640t, and 386–543 were created by site-directed mutagenesis, mutants 1–567, 1–611, c1–544, c1–642, and the deletion or point mutants of the EEVD residues were created using the polymerase chain reaction (PCR) and wild type Hsp70 or Hsc70 DNA as templates. The amplified DNA fragments were inserted into the vector, pGEX-2T (Pharmacia-LKB). The cloned fragments were digested with *Cla*I and *Eco*RI and inserted into the vector pMS1-543. The junctions of all deletion and point mutants in Hsp70 and Hsc70 were subjected to nucleotide sequence analysis. The HDJ-1 cDNA (Raabe and Manely, 1991) was shuttled into the pET-21d vector (Novagen) utilizing PCR, specific oligonucleotide primers and the wild type DNA. In addition, all DNA segments that were amplified by PCR were sequenced in their entirety to ensure that other mutations had not randomly occurred.

Protein purification

The various gene constructs were transformed into either DH-1, BL21/DE3 or BB1553 cells (Bukau and Walker, 1990). Bacterial transformants containing hsp70 or hdj-1 constructs were grown at 37°C (DH-1 or BL21/DE3) or 31°C (BB1553) whereas transformants containing hsc70 constructs were grown at 25°C (BL21/DE3). Protein extracts from IPTG-induced cells were prepared at 4°C and the recombinant proteins were purified by a combination of anion exchange and affinity chromatography. The crude extract was loaded onto a 200 ml DEAE fast flow Sepharose column (Pharmacia-LKB) and eluted with a 50–350 mM NaCl gradient over five column volumes. The fractions containing Hsp70 protein were pooled and recirculated over a 20 ml ATP–agarose column (Sigma; C-8 linkage) or 20 ml glutathione–agarose column (Pharmacia-LKB) washed with 2.0 M NaCl, equilibrated to 50 mM NaCl and eluted with 10 ml of column buffer containing 10% glycerol and either 50 mM Mg-ATP or 50 mM glutathione, respectively. The Hsp70-containing fractions were pooled, concentrated by ultrafiltration in a Centriprep-10 (Amicon), desalted over a G-25 column and dialyzed for 4 h in 2 l of 20 mM Tris (pH 6.9 r.t.), 0.1 mM EDTA and 100 mM NaCl. The protein sample was then loaded onto a 6 ml ResourceQ column (Pharmacia-LKB) and eluted with a 50–400 mM NaCl gradient over five column volumes. The fractions containing Hsp70 protein were pooled, concentrated by ultrafiltration in a Centricon-10 (Amicon) and dialyzed for 3 days against 20 mM Tris, pH 6.9, 0.1 mM EDTA, 100 mM NaCl at 4°C. Following dialysis, the protein concentrations were determined as an average of three measurements utilizing the BCA assay (Pierce) relative to a standard solution of bovine serum albumin (BSA). HDJ-1 was purified according to the protocol defined by Zylicz *et al.* (1985) for the purification of *E.coli* DnaJ.

RCMLA binding assays

Reduced carboxymethylated α-lactalbumin (RCMLA, Sigma) was radio-iodinated using carrier free $Na^{[125]}I$ (NEN, DuPont) and IodoBeads according to the manufacturer (Pierce). For the gel filtration experiments, Hsp70 (14 μM) and iodinated RCMLA were combined at a 5:1 molar ratio in buffer B (20 mM HEPES pH 7.2, 5 mM $MgCl_2$, 100 mM NaCl), incubated at 37°C for 30 min and resolved on a Superdex-200 gel filtration column (Pharmacia-LKB) in buffer B at 4°C, at 0.25 ml/min. Based upon the determined apparent K_d (9.5 μM) between wild type Hsp70 and RCMLA, a molar ratio of 5:1 Hsp70:RCMLA was utilized to acheive 50% RCMLA bound. The ^{125}I in each fraction was quantified in an IsoData 20/20 γ-counter.

The binding assays using native gel electrophoresis were performed with Hsp/Hsc70 proteins (14 μM final) and iodinated RCMLA that were combined at a 5:1 molar ratio in buffer B, incubated at 37°C for 30 min and resolved over a 6% acrylamide–1× TBE gel at 4°C.

ATPase assays

ATP hydrolysis was determined by measuring the release of $[^{32}P]P_i$ from $[\gamma\text{-}^{32}P]ATP$ according to the protocol of Sadis and Hightower (1992). The ATPase rates were determined utilizing an average $[\gamma\text{-}^{32}P]ATP$ hydrolysis rate at each time point (5, 10, 15 and 20 min) from three separate experiments for each Hsp70 protein after the background hydrolysis had been subtracted. The data were visualized and quantified by PhosphorImager analysis (Molecular Dynamics). The effect of a protein substrate (native α-lactalbumin or RCMLA) on the ATPase rate was measured in a 1:20 Hsp70:lactalbumin molar ratio prior to incubation at 37°C.

Limit proteolytic digestion in the presence of ATP or protein substrates

Proteolytic cleavage was carried out at 37°C by incubation of the various Hsp70 proteins (5 μg) with trypsin (0.005 μg) in buffer C with or without ATP (10 mM), α-lactalbumin or RCMLA. At the specified times samples were removed and the reactions were stopped by the addition of SDS sample buffer and boiling for 3 min. The samples were then resolved on SDS–PAGE, stained with Coomassie Blue and quantified by laser densiotometry (Pharmacia-LKB).

For Western blot analysis, the samples were analyzed on SDS–PAGE and electro-blotted to nitrocellulose. After blocking in 5% non-fat milk, the blots were probed with either the 3A3 monoclonal antibody, whose epitope is located between residues 503 and 524 of human Hsp70 (S.Murphy, personal communication), or the 5A5 monoclonal antibody, whose epitope is located between residues 124 and 264 of human Hsp70, in PBS, 0.1% Tween-20. After washing and probing with goat anti-mouse horseradish peroxidase conjugate in PBS, 0.1% Tween-20, the blot was rinsed and visualized by enhanced chemi-luminescence (Amersham ECL kit). The migration distance of each protein was used to calculate their respective masses, relative to protein standards electrophoresed on the same gel.

Luciferase renaturation assay

The refolding of guanidinium-HCl denatured firefly luciferase followed the protocol described by Buchberger *et al.* (1994). Firefly luciferase (Sigma) was prepared at 4 mg/ml in glycylglycine pH 7.4 and denatured by dilution into denaturation buffer (25 mM HEPES pH 7.5, 50 mM

KCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol and 6 M guanidine–HCl) for 30 min at 37°C. The luciferase activity reactivation assays were as follows. An aliquot of the denatured protein solution (312.5 ng in 1 μl total volume) was added to 124 μl of refolding buffer (25 mM HEPES pH 7.6, 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT and 5 mM ATP) supplemented with either HDJ-1 (1.6 μM) or an Hsp/Hsc70 protein (0.8 μM). At the specified times 1 μl of the refolding reaction was removed, added to 50 μl of refolding buffer and the luciferase activity was measured using a luminometer (Moonlight). The percent activities were calculated against 1 μl of luciferase which had been diluted to the same extent except only in refolding buffer.

Fluorescence emission spectra, gel filtration and glycerol gradient sedimentation

The fluorescence emission spectra were determined at 0.1 mg/ml in 20 mM HEPES, 5 mM $MgCl_2$ and 100 mM NaCl. The samples were excited at a wavelength of 295 nm and the emmission was monitored between 300 and 400 nm in an α-scan (Photon Technology International). The spectra presented are the smoothed average of three scans for each sample.

Gel filtration analysis was performed using a 100 μl (1 mg/ml) sample of each protein in buffer B and resolved over a Superdex-200 column (Pharmacia-LKB) at 0.25 ml/min equilibrated in buffer B at 22°C. A total of 25 0.5 ml fractions were collected, 12.5 μl of each fraction was resolved on a 10% SDS–PAGE and stained with Coomassie Blue. The elution volume for each protein was determined from the location of each protein's UV absorption peak (280 nm) relative to the void volume of the column and utilized to approximate the native size and Stokes' radius of each protein relative to standard curves. The standard curves were generated using known globular proteins of various molecular weights and Stokes' radii. A least-squares algorithm (KaleidaGraph) was used to fit a line through the standard data points to yield standard curves with correlation coefficients of R = 0.99.

For glycerol gradient analysis, a 100 μl (1 mg/ml) sample of each protein in buffer C was layered onto a 5.0 ml 10–30% (v/v) glycerol gradient in buffer C and centrifuged in a Beckman SW60Ti rotor at 35 000 r.p.m. for either 20 h at 22°C or 36 h at 4°C. Fractions (250 μl) were collected from the bottom of the gradient, 12.5 μl aliquots of each fraction were resolved on 13% SDS–PAGE and stained with Coomassie Blue. Two gradients containing protein standards (alcohol dehydrogenase, 7.4 S; bovine serum albumin, 4.3 S; cytochrome *c*, 1.9 S) were included in every experiment. The frictional ratio for each protein was determined utilizing the average sedimentation coefficient obtained from three separate gradients of each protein.

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References

Abravaya,K., Myers,M.P., Murphy,S.P. and Morimoto,R.I. (1992) *Genes Dev.*, **6**, 1153–1164.

Beckmann,R.P., Mizzen,L.A., and Welch,W.J. (1990) *Science*, **248**, 850–854.

Bhattacharyya,T., Karnezis,A.N., Murphy,S.P., Hoang,T., Freeman,B.C., Phillips,B. and Morimoto,R.I. (1995) *J. Biol. Chem.*, **270**, 1705–1710.

Bienz,M. (1984) *Proc. Natl Acad. Sci. USA*, **81**, 3138–3142.

Bresnick,E.H., Sanchez,E.R. and Pratt,W.B. (1988) *J. Steroid Biochem.*, **30**, 267–269.

Buchberger,A., Schroder,H., Buttner,M., Valencia,A. and Bukau,B. (1994) *Struct. Biol.*, **1**, 95–101.

Bukau,B. and Walker,G.C. (1990) *EMBO J.*, **9**, 4027–4036.

Caplan,A.J., Cyr,D.M. and Douglas,M.G. (1993) *Mol. Biol. Cell*, **4**, 555–563.

Chappell,T.G., Konforti,B.B., Schmid,S.L. and Rothman,J.E. (1987) *J. Biol. Chem.*, **262**, 746–751.

Chiang,H.-L., Terlecky,S.R., Plant,C.P. and Dice,J.F. (1989) *Science*, **246**, 382–385.

Chirico,W.J., Waters,M.G. and Blobel,G. (1988) *Nature*, **332**, 805–810.

Craig,E.A., Baxter,B.K., Becker,J., Halladay,J. and Ziegelhoffer,T. (1994) In Morimoto,R.I., Tissieres,A. and Georgopoulos,C. (eds), *The Biology of Heat Shock Proteins and Molecular Chaperones*. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 31–52.

Cyr,D.M., Xiangyang,L. and Douglas,M.G. (1992) *J. Biol. Chem.*, **267**, 20927–20931.

DeLuca-Flaherty,C., McKay,D.B., Parham,P. and Hill,B. (1990) *Cell*, **62**, 875–887.

Dworniczak,B. and Mirault,M.E. (1987) *Nucleic Acids Res.*, **15**, 5181–5197.

Flaherty,K.M., DeLuca-Flaherty,C. and McKay,D.B. (1990) *Nature*, **346**, 623–628.

Flajnik,M. F. Canel,C., Kramer,J. and Kasahara,M. (1991) *Immunogenetics*, **33**, 295–300.

Flynn,G.C., Chappell,T.G. and Rothman,J.R. (1991) *Science*, **245**, 385–390.

Frydman,J. and Hartl,F.-U. (1994) In Morimoto,R.I., Tissieres,A. and Georgopoulos,C. (eds), *The Biology of Heat Shock Proteins and Molecular Chaperones*. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 251–283.

Gao,Y., Thomas,J.O., Chow,R.L., Lee,G.H. and Cowan,N.J. (1992) *Cell*, **69**, 1043–1050.

Georgopoulos,C., Liberek,K., Zylicz,M. and Ang,D. (1994) In Morimoto,R.I., Tissieres,A. and Georgopoulos,C. (eds), *The Biology of Heat Shock Proteins and Molecular Chaperones*. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 209–249.

Giebel,L.B., Dworniczak,B.P. and Bautz,E.K.F. (1988) *Dev. Biol.*, **125**, 200–207.

Haus,U., Trommler,P., Fisher,P.R., Hartmann,H., Lottspeich,F., Noegel, A.A. and Schleicher,M. (1993) *EMBO J.*, **12**, 3763–3772.

Hightower,L.E., Sadis,S.E. and Takenaka,I.M. (1994) In Morimoto,R.I., Tissieres,A. and Georgopoulos,C. (eds), *The Biology of Heat Shock Proteins and Molecular Chaperones*. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 179–207.

Hunt,C. and Calderwood,S. (1990) *Gene*, **87**, 199–204.

Hunt,C. and Morimoto,R.I. (1985) *Proc. Natl Acad. Sci. USA*, **82**, 6455–6459.

Ingolia,T.D. and Craig,E.A. (1982) *Proc. Natl Acad. Sci. USA*, **79**, 525–529.

Kang,P.J., Ostermann,J., Shilling,J., Neupert,W., Craig,E.A. and Pfanner,N. (1990) *Nature*, **348**, 137–143.

Laloraya,S., Gambill,B.D. and Craig,E.A. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 6481–6485.

Lewis,V.A., Hunes,G.M., Zheng,D., Sibil,H. and Willison,K. (1992) *Nature*, **358**, 249–252.

Liberek,K. Skowyra,D., Zylicz,M., Johnson,C. and Georgopoulos,C. (1991a) *J. Biol. Chem.*, **266**, 14491–14496.

Liberek,K., Marszalek,J, Ang,D., Georgopoulos,C. and Zylicz,M. (1991b) *Proc. Natl Acad. Sci. USA*, **88**, 2874–2878.

Milarski,K.L. and Morimoto,R.I. (1986) *Proc. Natl Acad. Sci. USA*, **83**, 9517–9521.

Milarski,K.L. and Morimoto,R.I. (1989) *J. Cell Biol.*, **109**, 1947–1962.

Montgomery,D., Jordan,R., McMacken,R. and Freire,E. (1993) *J. Mol. Biol.*, **232**, 680–692.

Morimoto,R.I., Hunt,C., Huang,S.-Y., Berg,K.L. and Banerji,S.S. (1986) *J. Biol. Chem.*, **261**, 12692–12699.

Morimoto,R.I., Sarge,K.D. and Abravaya,K. (1992) *J. Biol. Chem.*, **267**, 21987–21990.

Munro,S. and Pelham,H. (1986) *Cell*, **46**, 291–300.

Murakami,H., Pain,D. and Blobel,G. (1988) *J. Cell Biol.*, **107**, 2051–2057.

Nelson,R.J., Ziegelhoffer,T., Nicolet,C., Werner-Washburne,M. and Craig,E.A. (1992) *Cell*, **71**, 97–105.

O'Malley,K., Mauron,A., Barchas,J.D. and Kedes,L. (1985) *Mol. Cell. Biol.*, **5**, 3476–3483.

Ostermann,J., Horwich,A.L., Neupert,W. and Hartl,F.U. (1989) *Nature*, **341**, 125–130.

Palleros,D.R. Welch,W.J. and Fink,A.L. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 5719–5723.

Palleros,D.R., Reid,K.L., Shi,L., Welch,W.J. and Fink,A.L. (1993) *Nature*, **365**, 664–666.

Raabe,T. and Manley,J.L. (1991) *Nucleic Acids Res.*, **19**, 6645.

Requena,J.M., Lopez,M.C., Jimenez-Ruyiz,A., De La Torre,J.C. and Alonso,C. (1988) *Nucleic Acids Res.*, **16**, 1393–1406.

Rochester,D.E., Winer,J.A. and Shah,D.M. (1986) *EMBO J.*, **5**, 451–458.

Sadis,S. and Hightower,L.E. (1992) *Biochemistry*, **31**, 9406–9412.

Shi,Y. and Thomas,J.O. (1992) *Mol. Cell. Biol.*, **12**, 2186–2192.
Skowyra,D., Georgopoulos,C. and Zylicz,M. (1990) *Cell*, **62**, 939–944.
Slater,M.R. and Craig,E.A. (1989) *Nucleic Acids Res.*, **17**, 805–806.
Smith,D.F., Faber,L.E. and Toft,D.O. (1990) *J. Biol. Chem.*, **265**, 3996–4003.
Stone,D.E. and Craig,E.A. (1990). *Mol. Cell. Biol.*, **10**, 1622–1632.
Tilly,K., McKittrick,N., Zylicz,M. and Georgopoulos,C. (1983) *Cell*, **34**, 641–646.
Ting,J. and Lee,A.S. (1988) *DNA*, **7**, 275–286.
Tsai,M-Y. and Chang,C. (1994) *J. Biol. Chem.*, **269**, 5958–5962.
Ungewickell,E. (1985) *EMBO J.*, **4**, 3385–3391.
Wu,B., Hunt,C. and Morimoto,R.I. (1985) *Mol. Cell. Biol.*, **5**, 330–341.
Yaffe,M.B., Farr,G.W., Miklos,D., Horwich,A.L., Sternlicht,M.L. and Sternlicht,H. (1992) *Nature*, **358**, 245–248.
Zylicz,M., Yamamoto,T., McKittrick,N., Sell,S. and Georgopoulos,C. (1985) *J. Biol. Chem.*, **260**, 7591–7598.

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